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INFECTION AND IMMUNITY, Nov. 2008, p. 5310–5321 Vol. 76, No. 11
0019-9567/08/$08.00+0 doi:10.1128/IAI.00960-08

Identification of *Salmonella enterica* Serovar Dublin-Specific Sequences by Subtractive Hybridization and Analysis of Their Role in Intestinal Colonization and Systemic Translocation in Cattle▽†

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Received 31 July 2008/Returned for modification 16 August 2008/Accepted 4 September 2008

***Salmonella enterica* serovar Dublin is a host-restricted serovar associated with typhoidal disease in cattle. In contrast, the fowl-associated serovar *S. enterica* serovar Gallinarum is avirulent in calves, yet it invades ileal mucosa and induces enteritis at levels comparable to those induced by *S. enterica* serovar Dublin. Suppression subtractive hybridization was employed to identify *S. enterica* serovar Dublin strain SD3246 genes absent from *S. enterica* serovar Gallinarum strain SG9. Forty-one *S. enterica* serovar Dublin fragments were cloned and sequenced. Among these, 24 mobile-element-associated genes were identified, and 12 clones exhibited similarity with sequences of known or predicted function in other serovars. Three *S. enterica* serovar Dublin-specific regions were homologous to regions from the genome of *Enterobacter* sp. strain 638. Sequencing of fragments adjacent to these three sequences revealed the presence of a 21-kb genomic island, designated *S. enterica* serovar Dublin island 1 (SDI-1). PCR analysis and Southern blotting showed that SDI-1 is highly conserved within *S. enterica* serovar Dublin isolates but rarely found in other serovars. To probe the role of genes identified by subtractive hybridization in vivo, 24 signature-tagged *S. enterica* serovar Dublin SD3246 mutants lacking loci not present in *Salmonella* serovar Gallinarum SG9 were created and screened by oral challenge of cattle. Though attenuation of tagged SG9 and SD3246 *Salmonella* pathogenicity island-1 (SPI-1) and SPI-2 mutant strains was detected, no obvious defects of these 24 mutants were detected. Subsequently, a ΔSDI-1 mutant was found to exhibit weak but significant attenuation compared with the parent strain in coinfection of calves. SDI-1 mutation did not impair invasion, intramacrophage survival, or virulence in mice, implying that SDI-1 does not influence fitness per se and may act in a host-specific manner.**

There are over 2,500 different serovars of *Salmonella enterica*, and some are significant pathogens of animals and humans. All *S. enterica* serovars are closely related, and comparisons of housekeeping genes show 96 to 99.5% sequence identity (reviewed in reference 13). Although *S. enterica* serovars are genetically very similar, they differ significantly in biology, particularly in host range and disease spectrum. *S. enterica* serovars may be broadly classified as ubiquitous, host restricted, and host specific (41). In healthy, adult, outbred hosts, ubiquitous serovars, including *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis, are frequently associated with self-limiting intestinal infections in a wide range of phylogenetically distantly related species (38, 43). Host-specific serovars are almost exclusively associated with typhoidal disease in a single species, for example, *S. enterica* serovar Typhi and *S. enterica* serovar Gallinarum in humans and fowl, respectively (2, 12). Serovars which are predominantly isolated from one particular host species but which occasionally cause disease in other host species are classified as host restricted; for example, *S. enterica* serovar Dublin is associated with cattle (38) but sometimes infects pigs and humans. In general, host-specific and host-restricted serotypes tend to be more virulent, causing systemic disease and causing higher mortality rates than ubiquitous serotypes (reviewed in references 3 and 41). Survivors of systemic salmonellosis sometimes become chronic carriers, thereby facilitating bacterial circulation in host populations (21).

Analysis of the genetic differences responsible for the phenotypic diversity among serovars is currently a major area of *Salmonella* research. Host restriction has occurred by convergent evolution in some instances, as there are cases in which no close phylogenetic relationship exists between serovars adapted to the same host, for example, *S. enterica* serovar Typhi and other human-restricted serovars (24, 35). Conversely, serotypes that are genetically closely related may be adapted to different hosts, for example, *S. enterica* serovar Choleraesuis and *S. enterica* serovar Paratyphi C (35). Adaptation to a particular host species is a complex process that may involve both the acquisition of serovar-specific sequences by lateral gene transfer and gene decay. A number of serovar-specific insertions, deletions, and frameshift mutations have been described previously (4, 15, 24, 26, 30, 39, 40, 46). For example, sequence analysis of the fimbrial genes in several serovars shows that many of the serovars contain frameshifts in one or several of the operons (reviewed in reference 13). Since fimbrial adhesins are involved in interactions with different receptors, this diversity could influence host specificity. Among

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† Supplemental material for this article may be found at http://iai.asm.org/.

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▽ Published ahead of print on 15 September 2008.

the best characterized of the serovar-specific islands acquired by horizontal transfer is *Salmonella* pathogenicity island-7 (SPI-7) in *S. enterica* serovar Typhi, which encodes the Vi capsular antigen, which is absent from most other serovars (29).

The aim of this study was to investigate the genetic basis of the differential virulence of *S. enterica* serovar Dublin and *S. enterica* serovar Gallinarum in cattle. Previously, we have reported that *S. enterica* serovar Dublin strain SD3246 elicited severe systemic disease following oral inoculation of calves, whereas *S. enterica* serovar Gallinarum strain SG9 was avirulent by this route (28). Differential virulence was not correlated with intestinal invasion or the induction of enteritis (28) but correlated with increased persistence of *S. enterica* serovar Dublin in intestinal mucosa (28) and the ability of *S. enterica* serovar Dublin to translocate to distal sites via the lymphatic system (33). Though the role of known or putative virulence loci in systemic translocation has been assessed (33), traits associated with the differential virulence of *S. enterica* serovar Dublin SD3246 compared to other serovars remain ill defined. It was recently reported that the virulence plasmid of *S. enterica* serovar Dublin contains a unique 10.8-kb region that is absent from the plasmids of *S. enterica* serovars Choleraesuis, Enteritidis, and Typhimurium and contains 16 potential open reading frames (ORFs) (20). We have previously screened 120 mutants with transposon insertions in this unique region of the *S. enterica* serovar Dublin virulence plasmid (SacI fragments C and F), and only one mutant (G19) exhibited reduced virulence for mice (22). The transposon insertion in G19 was in *vagC* and led to uncontrolled expression of the downstream gene *vagD* (32). Other transposon mutants with insertions in *vagC* were fully virulent (32). Thus, it is unlikely that the other genes on this *S. enterica* serovar Dublin-specific plasmid region are required for virulence. A previous microarray study identified DNA sequences that were present in *S. enterica* serovar Typhimurium, Typhi, Paratyphi A, or Enteritidis but absent from either *S. enterica* serovar Dublin or *S. enterica* serovar Gallinarum (30). The significance of these deleted sequences for *S. enterica* serovar Dublin and *S. enterica* serovar Gallinarum is unknown. *S. enterica* serovar Dublin-specific chromosomal regions have not been previously identified. As the genome sequence of *S. enterica* serovar Dublin SD3246 is unknown, we used suppression subtractive hybridization to identify and analyze *S. enterica* serovar Dublin SD3246 chromosomal genes that are absent from *S. enterica* serovar Gallinarum SG9.

MATERIALS AND METHODS

Bacterial strains. *S. enterica* serovar Dublin SD3246 (18) and *S. enterica* serovar Gallinarum SG9 (45) were isolated from cases with bovine and fowl typhoid, respectively. *S. enterica* serovar Dublin SD3246 is a Vi antigen-negative isolate, and a nalidixic acid-resistant (Nal^r) derivative with defined virulence in cattle was used (5, 28, 33). Virulent signature-tagged derivatives of these strains and tagged SD3246 mutants lacking SPI-1 and SPI-2 genes have been described previously (33). Another 71 wild-type *Salmonella* isolates were used in this study: these included strains from the United Kingdom, isolated from animals at the Institute for Animal Health, Compton, isolates obtained from the Veterinary Laboratories Agency, United Kingdom, and isolates from *Salmonella* reference collection B (SARB) (6). These consisted of 31 different serovars and subspecies of *S. enterica*, namely, serovars Dublin (14 isolates), 45:a;enx (1), Agama (1), Agona (1), Anatum (1), Brandenburg (2), Choleraesuis (5), Choleraesuis variant Decatur (2), Derby (3), Duisburg (2), Emek (2), Enteritidis (4), Haifa (1), Heidelberg (2), Indiana (1), Infantis (2), Miami (2), Montevideo (2), Muenchen (3), Newport (2), Panama (1), Pullorum (1), Reading (1), Rubislaw (1), Saintpaul (1), Senftenberg (1), Stanley (1), Stanleyville (1), Typhimurium (7), and Wien (2) and *S. enterica* subsp. *diarizonae* (1). All strains were stored as mid-log-phase cultures in Luria-Bertani (LB) medium containing 15% (vol/vol) glycerol at −70°C. Unless otherwise stated, strains were cultured in LB medium at 37°C with the antibiotics ampicillin (100 $\mu g\ ml^{-1}$), kanamycin (Kan; 50 $\mu g\ ml^{-1}$), and Nal (20 $\mu g\ ml^{-1}$) where appropriate.

General molecular techniques. Restriction enzymes, GoTaq DNA polymerase, and T4 DNA ligase were purchased from Promega Corporation (Southampton, United Kingdom) or New England Biolabs (Hertfordshire, United Kingdom) and used according to the manufacturer's recommendations. Oligonucleotide primers were obtained from Sigma Genosys (Poole, United Kingdom) (see Table S1 in the supplemental material). PCR products for sequencing were purified by using QiaQuick PCR purification kits (Qiagen, Crawley, United Kingdom). Genomic DNA from *Salmonella* was prepared by cetyltrimethyl ammonium bromide extraction as described previously (37). DNA probes for Southern hybridization consisted of digoxigenin-labeled PCR products amplified from strain SD3246 with the digoxigenin DNA labeling and detection kit supplied by Roche Molecular Biochemicals (Mannheim, Germany).

Subtractive hybridization. Subtractive hybridization was performed by using *S. enterica* serovar Dublin SD3246 genomic DNA as the tester. Driver DNA contained a mix of *S. enterica* serovar Gallinarum SG9 genomic DNA and *S. enterica* serovar Dublin SD3246 plasmid DNA. Both DNA samples were digested with RsaI. The procedure was carried out by using the Clontech PCR-Select bacterial genome subtraction kit (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions. PCR products were cloned into pGEM-T Easy (Promega Corporation, Southampton, United Kingdom) and transformed into chemically competent *Escherichia coli* JM109 cells (Promega Corporation, Southampton, United Kingdom).

Construction of signature-tagged *S. enterica* serovar Dublin SD3246 mutants. Uniquely tagged mini-Tn*5*Km2 mutants of *S. enterica* serovar Dublin SD3246 Nal^r with insertions in the sequences identified by subtractive hybridization were created by targeted lambda red recombinase-mediated integration of linear PCR products (11). Compatible tagged transposons were amplified by PCR using primers which incorporate 40-nucleotide gene-specific homology extensions, designed to replace an internal part of the sequence of interest with the transposon. Products were DpnI digested, purified using QiaQuick spin columns (Qiagen, Crawley, United Kingdom) and electroporated into *S. enterica* serovar Dublin SD3246 Nal^r harboring the lambda red helper plasmid pKD46 following induction of the recombinase with 0.2% (wt/vol) L-arabinose at 30°C (11). Mutants were selected on LB plates containing Nal and Kan at 37°C and cured of pKD46 by growth at 37°C in the absence of ampicillin. Transposon insertion sites were confirmed by PCR analysis. An *S. enterica* serovar Dublin SD3246 mutant with a deletion spanning *phoPQ* was also created by this method with the use of primers (see Table S1 in the supplemental material) as a control for intramacrophage survival assays.

DNA sequencing and analysis. DNA sequencing reactions were performed using the Quickstart kit (Beckman Coulter, High Wycombe, United Kingdom). For the sequencing of the inserts in the subtractive hybridization library, M13For and M13Rev primers were used (see Table S1 in the supplemental material). Sequencing reactions were run on a Beckman-Coulter CEQ8000 sequencer. The BLASTX and BLASTN programs were used to search the NCBI nonredundant sequence database (http://www.ncbi.nlm.nih.gov) and the COLIBASE database (http://colibase.bham.ac.uk) to identify sequence similarities. GLIMMER version 3.02 (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi) was used to predict coding sequences, and InterProScan (http://www.ebi.ac.uk/InterProScan) was used to predict protein domains.

Calf experiments. Animal experiments were conducted according to the requirements of the Animal (Scientific Procedures) Act 1986 (license 30/1998) with the approval of the local ethical review committee. Friesian bull calves were reared, housed, and confirmed to be culture negative for *Salmonella* as described previously (28). Calves 25 to 35 days of age were used for infection experiments.

For the screening of a pool of 36 signature-tagged mutants following oral inoculation of calves, mutants were separately inoculated into LB broth supplemented with Kan and Nal and incubated overnight at 37°C. The mutants were pooled, and an aliquot was removed for preparation of "input pool" genomic DNA as described previously (19). Two calves were orally inoculated with the pool (1.8×10^9 CFU per calf) in 20 ml antacid [5% (wt/vol) $Mg(SiO_3)_3$, 5% (wt/vol) $NaHCO_3$, and 5% (wt/vol) $MgCO_3$ in H_2O]. Calves were anesthetized at 3 days postinoculation, the distal ileal loop was exteriorized, and an efferent lymph vessel draining the loop was cannulated as described previously (28). Lymph was collected for 3 to 4 h into heparinized tubes. Lymph samples and

homogenates of tissue collected at necropsy (distal ileal mucosa, draining mesenteric lymph node [MLN], liver, and spleen) were diluted as required and plated onto MacConkey agar containing Kan and Nal to isolate "output pool" bacteria. A sample of jugular blood collected during the cannulation was similarly plated. For each site, ca. 2,500 to 12,000 colonies were pooled for preparation of output pool genomic DNA. Amplification of radiolabeled tags from input and output pools and dot blot hybridizations were performed as described previously (25).

For the determination of competitive indices (CIs) in vivo, bacterial strains were grown in LB broth supplemented with Nal overnight at 37°C. Wild-type and mutant strains were mixed in equal numbers (ca. 1.5×10^9 CFU per calf) in 20 ml antacid and used to inoculate a total of six calves by the oral route (three independent experiments with two calves per experiment). At 3 days postinoculation, an efferent lymphatic vessel was cannulated and lymph was collected as described above. Wild-type and mutant bacteria were enumerated by plating of serial dilutions of the lymph or homogenized tissue samples collected at necropsy onto MacConkey agar with Nal and with Nal plus Kan. The number of wild-type bacteria was determined by subtraction of the count on Nal and Kan medium (mutant) from that on Nal alone. The CI was calculated as the ratio of mutant to wild type in the output pool divided by the ratio of mutant to wild type in the inoculum. Data are presented as the mean CIs ± standard errors of the means. The Mann-Whitney nonparametric test was used to determine whether the output ratio differed significantly from the input ratio. P values of <0.05 were considered significant.

Mouse experiments. For the determination of CIs in mice, bacteria were grown in LB broth supplemented with Nal overnight at 37°C. Wild-type and mutant strains were mixed in equal numbers, and 16 female C57BL/6 mice (6 to 8 weeks of age) were infected by the oral route via a gavage needle with approximately 2×10^6 CFU diluted in phosphate-buffered saline (PBS). Animals were examined at least twice daily. Mice showed symptoms of systemic salmonellosis after 3 to 6 days, at which time they were humanely killed. Spleens and livers were removed, each organ was homogenized in PBS, and serial dilutions of these suspensions were plated to enumerate wild-type and mutant strains as described above.

Determination of in vitro CIs. Bacterial strains were grown in LB broth supplemented with Nal overnight at 37°C. *S. enterica* serovar Dublin SD3246 Nal^r and mutant strains were mixed in equal numbers (ca. 10^4 CFU per ml) in three 10-ml volumes of LPM minimal medium (7) containing Nal and 50 μg/ml nicotinic acid and incubated at 37°C at 130 rpm for 24 h. Wild-type and mutant bacteria were enumerated by plating of serial dilutions of these output cultures and the input cultures and the CI calculated as described above.

Cultured cell assays of invasion, intracellular growth, and survival. Invasion, intracellular growth, and survival of *Salmonella* strains were assayed in INT407 (also known as Henle 407) cells, a human intestinal epithelial cell line. INT407 cells were seeded at 5×10^5 cells per well in Eagle's minimal essential medium supplemented with 10% (vol/vol) fetal calf serum (complete EMEM) in 24-well plates. The plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h to obtain confluent monolayers. The monolayers were washed once with PBS, and 0.5-ml volumes of complete EMEM were added to each well 30 min prior to the addition of bacteria. Bacterial strains were grown overnight at 25°C at 130 rpm, then subcultured 1:10 into fresh LB broth without antibiotics, and incubated at 37°C at 130 rpm for 90 min. The inocula were diluted to approximately 1×10^6 CFU/ml in complete EMEM, and 0.5 ml of diluted bacteria was added to each well, in triplicate, for each strain. After incubation for 1 h, the monolayers were washed three times with PBS and incubated for a further hour in complete EMEM containing 100 μg ml^{-1} gentamicin. The monolayers were then washed once with PBS, and the medium was replaced with fresh complete EMEM containing 10 μg ml^{-1} gentamicin. At different time points, monolayers were washed three times with PBS and lysed by the addition of 100-μl volumes of PBS containing 1% (vol/vol) Triton X-100 per well. After 5 min at room temperature, 0.9-ml volumes of PBS were added, and bacteria were enumerated by plating suitable dilutions onto LB agar plates containing appropriate antibiotics. In some experiments, samples of the culture medium were removed from each well for the determination of cytotoxicity. Cytotoxicity was determined using the CytoTox 96 nonradioactive cytotoxicity assay (Promega Corporation, Southampton, United Kingdom), which quantitates lactate dehydrogenase, a stable cytosolic enzyme that is released upon cell lysis.

Bacterial survival was also assayed in J774 murine macrophage-like cells. J774 cells were seeded at 2×10^5 cells per well in 24-well plates on poly-L-lysine-coated coverslips in RPMI medium containing 10% (vol/vol) fetal calf serum (complete RPMI). After 24-h incubation at 37°C in a humidified 5% CO_2 atmosphere, macrophages were activated as described previously (1). Briefly, the medium was replaced with complete RPMI containing 0.1 μg/ml of lipopolysaccharide (from *E. coli* O55:B5; Sigma, Poole, United Kingdom) and cells were incubated for a further 24 h. Bacterial strains were grown in LB broth at 37°C to stationary phase and diluted to 2×10^7 CFU/ml in sterile PBS. Diluted bacterial suspensions (200-μl volumes) were added to each well, in triplicate, and the plates were incubated for 30 min. Monolayers were then washed three times with PBS, and fresh complete medium containing 100 μg/ml gentamicin was added to the wells. At different time points after infection, cells were washed and lysed, and bacteria were enumerated as described for the INT407 experiments.

The results of the cell assays were analyzed using Student's t test (paired). P values of <0.05 were considered significant.

Mitomycin C induction. *S. enterica* serovar Dublin wild-type strains and *S. enterica* serovar Typhimurium 4/74 were grown overnight in 10-ml volumes of LB at 37°C at 130 rpm before being subcultured 1:25 into four 50-ml volumes of LB and growing to an optical density at 600 nm (OD_{600}) of 0.3 to 0.4. Mitomycin C was added to the exponentially growing cultures to a final concentration of 300 ng ml^{-1}, 1 μg ml^{-1}, or 2 μg ml^{-1}, and incubation continued at 37°C at 130 rpm. Control cultures without mitomycin C were included. Samples were taken at 30-min intervals for 6 h after mitomycin C addition, and the OD_{600} was measured. A drop in OD_{600} indicated that phage induction causing bacterial cell lysis had occurred. When no reduction in absorbance occurred, incubation was continued overnight, before the OD_{600} was measured again. In some experiments, the cultures were lysed 3 to 5 h after mitomycin C addition with 2% (vol/vol) chloroform, and the incubation continued for 15 min at 37°C at 130 rpm. Cell debris was removed by centrifugation for 10 min at 15,000 × g, and the supernatants were filtered through 0.45-μm filters and stored at 4°C. Phage DNA purification was attempted using lambda midi kits (Qiagen, Crawley, United Kingdom).

Nucleotide sequence accession numbers. The nucleotide sequences of the 41 *S. enterica* serovar Dublin SD3246 fragments described here which are absent in *S. enterica* serovar Gallinarum SG9 have been deposited in the GenBank dbGSS database and assigned accession numbers ET634245 to ET634285. The sequence of the 26,210-bp *S. enterica* serovar Dublin SD3246 fragment containing *S. enterica* serovar Dublin island 1 (SDI-1) has been deposited in GenBank under accession number EU624320.

RESULTS

Construction and sequence analysis of a library of *S. enterica* serovar Dublin SD3246 sequences absent from *S. enterica* serovar Gallinarum SG9. Preliminary results of experiments using total DNA from these two strains to produce a subtractive hybridization library showed that most products contained *S. enterica* serovar Dublin SD3246 virulence plasmid genes (data not shown). Since *S. enterica* serovar Dublin-specific plasmid genes are well studied (20, 22, 33), we prepared another subtractive hybridization library by including SD3246 plasmid DNA in the driver DNA sample to identify chromosomal genes present in *S. enterica* serovar Dublin SD3246 but not SG9. Sequence analysis and BLASTN searches of this library identified 51 clones containing inserts with no significant sequence similarity to the sequenced *S. enterica* serovar Gallinarum strain (strain 287/91), of which 41 were unique (Table 1).

The COLIBASE database contains sequence data from a partially sequenced *S. enterica* serovar Dublin strain, CT02021853. The G+C content of this strain is 52.2%. BLASTN searches using the 41 fragments showed that they all had close homologues in CT02021853 (Table 1). The sizes of the 41 subtractive hybridization products ranged from 248 to 1,023 bp, and their G+C contents ranged from 33.9 to 55.6%.

The putative functions of proteins encoded by genes in the subtractive hybridization library were investigated using BLASTX, and many of them were associated with mobile genetic elements, including phage proteins, a transposase, and recombination hot spot (RHS) elements (Table 1). The phage-related genes (D21 to D41) had G+C contents ranging from

TABLE 1. Subtractive hybridization products present in *S. enterica* serovar Dublin SD3246 but not in *S. enterica* serovar Gallinarum SG9

Clone	Insert size (bp)	G+C content (%)	Best BLASTX hit(s) in NCBI		E value	Accession no.	BLASTN hits for other selected salmonellae (E values < 1e−50)[a]
			Protein function	Source			
D1	384	40.4	Putative methyl-accepting chemotaxis protein	*S. enterica* serovar Choleraesuis SC-B67	4e−58	YP_216282	A-H, J-L
D2	699	48.5	Putative serine protein kinase	*S. enterica* serovar Choleraesuis SC-B67	2e−96	YP_216284	A-L
D3	462	53.2	Putative NAD-dependent aldehyde dehydrogenase	*S. enterica* serovar Typhimurium LT2	1e−45	NP_463378	A-I, K, L
D4	668	44.5	Putative flagellin structural protein	*S. enterica* serovar Paratyphi A ATCC 9150	6e−111	YP_151546	E, L
D5	403	42.7	Putative flagellin structural protein	*S. enterica* serovar Paratyphi A ATCC 9150	6e−68	YP_151546	E, L
D6	440	45.2	Hypothetical protein	*S. enterica* serovar Typhi CT18	2e−53	NP_455838	A-I, K, L
D7	419	55.6	Putative cytoplasmic protein	*S. enterica* serovar Typhimurium LT2	6e−68	NP_461942	A-J, L
D8	509	47.9	Putative amino acid transport protein	*S. enterica* serovar Paratyphi A ATCC 9150	1e−79	YP_152044	A-L
D9	305	43.3	Putative transport protein	*S. enterica* serovar Typhimurium LT2	2e−53	NP_460503	A-C, F-H, K, L
D10	423	44.2	Hypothetical protein	*Enterobacter* sp. strain 638	1e−05	YP_001175784	H, I, L
D11	665	49.2	Hypothetical protein	*Enterobacter* sp. strain 638	9e−43	YP_001175781	L
D12	901	47.8	Hypothetical protein	*Enterobacter* sp. strain 638	2e−70	YP_001175779	L
D13	315	49.2	Putative inner membrane protein	*S. enterica* serovar Typhimurium LT2	1e−32	NP_459281	A-G, L
D14	662	34.3					L
D15	835	35.4	Hypothetical protein	*Methanosarcina mazei*	4e−28	NP_634583	L
D16	820	33.9					L
D17	517	41.0	Hypothetical protein	*S. enterica* serovar Typhi CT18	6e−30	NP_454904	B, C, L
D18	393	39.7	Rhs family protein	*S. enterica* serovar Paratyphi A ATCC9150	2e−12	YP_151659	L
D19	877	50.7	Rhs family protein	*S. enterica* serovar Typhi CT18	1e−99	NP_454896	A-C, F, G, L
D20	357	51.0	Transposase	*S. enterica* serovar Choleraesuis SC-B67	9e−16	YP_215992	A, D, F-I, L
D21	670	49.7	Hypothetical protein (Fels-2 prophage)	*S. enterica* serovar Typhimurium LT2	1e−38	NP_461662	A-C, E, I, L
D22	383	52.2	Probable capsid portal protein	*S. enterica* serovar Typhi CT18	2e−69	NP_457865	A-C, E, G, I, J, L
D23	576	51.9	Probable bacteriophage replication endonuclease	*S. enterica* serovar Paratyphi A ATCC9150	5e−95	YP_151769	A-C, E, G, I, L
D24	251	54.2	Protein gp55 precursor (bacteriophage)	*S. enterica* serovar Choleraesuis SC-B67	1e−21	YP_216199	D, K, L
D25	434	49.8	Terminase large subunit (phage ST64B)	*S. enterica* serovar Typhimurium DT64	7e−79	NP_700375	F, G, L
D26	568	45.2	Phage holin	*S. enterica* serovar Paratyphi A ATCC9150	1e−09	YP_151591	E, F, L
D27	277	41.2	Antitermination protein Q (phage V)	*Shigella flexneri*	2e−10	NP_599078	F, H, L
D28	282	48.2	Antitermination protein Q (phage V)	*Shigella flexneri*	2e−47	NP_599078	F, H, L
D29	569	53.8	Phage-encoded protein	*E. coli* E22	2e−70	ZP_00726849	D, F-H, L
D30	418	48.6	Putative methyltransferase	*S. enterica* serovar Typhi CT18	4e−17	NP_455505	L
D31	682	49.7	Portal protein (phage ST64T)	*S. enterica* serovar Typhimurium DT64	3e−128	NP_720327	E, F, L
D32	289	49.8	Putative antirepressor (phage cdtI)	*E. coli*	4e−46	YP_001272557	D, F-H, L
D33	1,023	50.7	RusA (resolvase)	*S. enterica* serovar Choleraesuis SC-B67	9e−51	YP_215331	D-F, L
D34	610	46.1	Nin-like protein (bacteriophage)	*S. enterica* serovar Choleraesuis SC-B67	4e−115	YP_215329	D, L
D35	281	51.6	Coat protein (phage ST64T)	*S. enterica* serovar Typhimurium DT64	2e−45	NP_720329	E, F, L
D36	248	51.6	Exodeoxyribonuclease VIII (Gifsy-1 phage)	*S. enterica* serovar Choleraesuis SC-B67	3e−42	YP_217625	A, D, F, G, L
D37	666	52.0	Eae-like protein (phage epsilon)	*S. enterica*	1e−62	NP_848252	E, F, L
D38	754	53.6	Putative chitinase (phage)	*S. enterica* serovar Typhi CT18	1e−91	NP_455520	A-D, F-H, K, L
D39	934	49.6	Hypothetical phage protein	*S. enterica* serovar Typhi CT18	6e−96	NP_455506	B, C, E, G, L
D40	568	48.9	Probable regulatory protein (phage Gifsy-2)	*S. enterica* serovar Typhimurium LT2	4e−104	NP_459989	A-D, F, G, L
D41	517	52.8	Major capsid protein precursor	*S. enterica* serovar Typhimurium DT64	2e−69	NP_700379	F, G, L

[a] The classifications of other *Salmonella* serovars with completed genome sequences were as follows: A, *S. enterica* serovar Typhimurium LT2; B, *S. enterica* serovar Typhi CT18; C, *S. enterica* serovar Typhi Ty2; D, *S. enterica* serovar Choleraesuis SC-B67; and E, *S. enterica* serovar Paratyphi A ATCC9150. The classifications of serovars with unfinished genome sequences (available at COLIBASE) are as follows: F, *S. enterica* serovar Typhimurium DT104; G, *S. enterica* serovar Typhimurium SL1344; H, *S. enterica* serovar Enteritidis PT4; I, *S. enterica* serovar Enteritidis LK5; J, *S. enterica* serovar Pullorum; K, *S. bongori*; and L, *S. enterica* serovar Dublin CT02021853. Analysis was carried out during June 2007.

41.2 to 54.2% (Table 1). Most of these genes were highly similar to prophage genes from other *S. enterica* prophages, including the *S. enterica* serovar Typhimurium prophages Fels-2, Gifsy-2, ST64B, and ST64T.

Seventeen of the subtractive hybridization products were not homologous to known phages or other mobile elements (clones D1 to D17) (Table 1). Six were similar to hypothetical proteins, two showed no significant similarities (the D14 and

D16 products), and nine were similar to proteins with known or predicted functions. Most of the last group were present in a number of *Salmonella* serovars. However, clones D4 and D5 contained inserts that were similar to those of *S. enterica* serovar Paratyphi A strain ATCC 9150 but were absent from all other sequenced salmonellae except serovar Dublin. The proteins were similar to different regions of the same protein, a putative flagellin structural protein.

Analysis of the distribution of the 41 sequences among sequenced salmonellae using BLASTN showed that seven subtractive hybridization clones (D11, D12, D14, D15, D16, D18, and D30) were present in *S. enterica* serovar Dublin only, suggesting they might contain sequences unique to *S. enterica* serovar Dublin. One of these, clone D30, potentially encodes a methyltransferase present on a phage. D18 potentially encodes an RHS element protein and had a relatively low G+C content (39.7%). The two clones whose translated products showed no protein similarities by BLASTX were also potentially specific to *S. enterica* serovar Dublin and had low G+C contents (34.3% for D14 and 33.9% for D16). BLASTN analysis of clone D16 in COLIBASE (carried out in December 2007) showed the sequence was present in the *S. enterica* serovar Dublin CT02021853 contig ABAP01000045. The sequences flanking the D16 region are similar to RHS-like family genes, suggesting that the D16 sequence is a novel sequence inserted into an RHS element. The sequence of clone D14 was present in *S. enterica* serovar Dublin contig ABAP01000018, which contains genes that are highly homologous to those of the *S. enterica* serovar Typhimurium LT2 Fels-2 phage. The D14 sequence is located between homologues of the STM2710 and STM2711 genes. Another potentially *S. enterica* serovar Dublin-specific clone (D15) encoded a product similar to a hypothetical protein from *Methanosarcina mazei*. Clone D15, which had a low G+C content (35.4%), was also present on contig ABAP01000018, adjacent to a homologue of the *S. enterica* serovar Typhimurium LT2 Fels-2 phage gene STM2723. Therefore, the *S. enterica* serovar Dublin-specific sequences in clones D14 and D15 are both inserted into a Fels-2-like prophage.

The other potentially *S. enterica* serovar Dublin-specific clones (D11 and D12) encoded products with similarity to hypothetical proteins from *Enterobacter* sp. strain 638. Clone D10 also encoded a protein with similarity to a hypothetical protein from *Enterobacter* sp. strain 638, though this was also present in *S. enterica* serovar Enteritidis strains PT4 and LK5. The *Enterobacter* homologues were analyzed further (see below).

Identification and analysis of SDI-1. As described above, clones D10 to D12 potentially encoded proteins that were similar to *Enterobacter* sp. strain 638 proteins. The level of amino acid identity ranged from 39 to 75%. Interestingly, the three *Enterobacter* proteins were encoded by genes located close to each other on a region of about 4 kb. In the unfinished *S. enterica* serovar Dublin genome sequence available at this time, the D10 to D12 sequences were on short, contiguous sequences (contigs 2134 and 2241). Contig 2241 was 2,699 bp, and one end of it was highly homologous to phage tail fiber genes, including *S. enterica* serovar Typhimurium LT2 STM1049, which encodes the Gifsy-2 phage tail protein (99% nucleotide identity over 928 bp). To determine whether the similarity between *S. enterica* serovar Dublin and *Enterobacter* sp. strain 638 extended further than this 4 kb, the sequences of adjacent *Enterobacter* ORFs were compared with translated sequences in COLIBASE. The *Enterobacter* proteins Ent638_1030 to Ent638_1051 were similar to the products of 10 translated *S. enterica* serovar Dublin contigs, suggesting that there was a sequence of about 20 kb of similar organization to an *Enterobacter* region located adjacent to a phage tail fiber gene. This region showed no nucleotide or amino acid sequence similarity to any other sequenced *Salmonella* strain, suggesting that it could be an *S. enterica* serovar Dublin-specific genomic island. Ent638_1052 is a phage tail assembly chaperone and was similar to a number of *Salmonella* phage tail-associated proteins.

In order to amplify across the gaps between the 10 *S. enterica* serovar Dublin contigs, PCR primers were designed to anneal near both ends of these contig sequences (see Table S1 in the supplemental material). PCR fragments were amplified from *S. enterica* serovar Dublin SD3246 genomic DNA by using the appropriate primer pairs, which showed that sequences similar to these contigs are present in SD3246 and that they link to form a large island. The complete DNA sequence of this *S. enterica* serovar Dublin SD3246 region was determined. However, as described above, only one boundary of the *S. enterica* serovar Dublin-specific island had been located. The required flanking region was obtained by using lambda red mutagenesis to specifically insert a mini-Tn*5*KmR transposon near the end of the island (Materials and Methods) and then cloning restriction fragments conferring kanamycin resistance from this mutant. In this way, a further ca. 6-kb region was cloned and its sequence was determined. The nucleotide sequence of most of this was highly homologous to those of several *Salmonella* serovars.

In total, a sequence of 26,210 bp was determined. BLASTN analysis of this sequence showed that only nucleotides 1 to 4561 and 25729 to 26210 are present in other sequenced salmonellae, with the central region of approximately 21 kb potentially being specific to *S. enterica* serovar Dublin. This novel region was designated SDI-1. SDI-1 has a G+C content of 51.3%, which is very similar to that of the *S. enterica* serovar Dublin genome. Since this work, the *S. enterica* serovar Dublin CT02021853 genome sequencing project has progressed, and the small contigs available then have now been assembled into larger contigs. Analysis of the updated genome sequence data shows that *S. enterica* serovar Dublin CT02021853 contains a corresponding island on contig ABAP01000038. There are only two nucleotide differences between the 26,210-bp regions of the two strains, with just one of these being within SDI-1.

The coding sequences of the 26,210-bp sequence were predicted using GLIMMER (Fig. 1 and Table 2). All the predicted ORFs are on the same strand. BLASTP and InterProScan were used to analyze the potential roles and functional domains of these proteins (Table 2). Products of ORF1 to ORF8, ORF10, ORF11, and part of ORF32 were predicted to be prophage proteins, while the ORF9 protein had no significant homologues. The other ORF proteins were most similar to *Enterobacter* sp. strain 638 proteins with unknown functions, except for Ent638_1033, which is predicted to be a NUDIX hydrolase. The lengths of the ORFs corresponded closely but not always exactly between *S. enterica* serovar Dublin and *Enterobacter*. The *Enterobacter* protein Ent638_1049 is predicted to contain a ubiquitin-activating enzyme 1 (E1) domain. Inter-

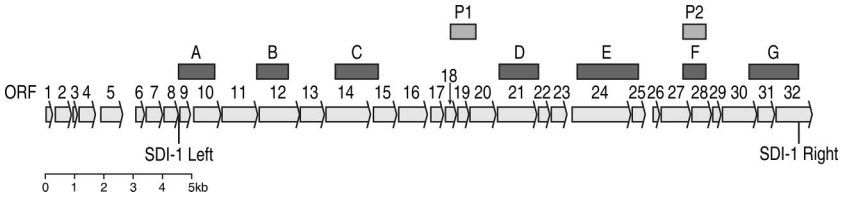

FIG. 1. Schematic representation of the *S. enterica* serovar Dublin SD3246 26,210-bp region, including the novel island SDI-1. The boundaries of the island are indicated. The position, orientation, and numbering of the ORFs are shown by arrows. The positions of the regions amplified by PCR to determine the distribution of the island among other serovars (A to G) and the probes used in Southern hybridizations (P1 and P2) are indicated by dark and light gray horizontal bars, respectively.

estingly this domain appears to be deleted from the corresponding *S. enterica* serovar Dublin ORF (ORF30). *S. enterica* serovar Dublin ORF27 has an N-terminal proline-rich 11-residue insertion which is not in Ent638_1046.

The addition of mitomycin C to cultures of *S. enterica* serovar Dublin SD3246 did not lead to prophage induction under conditions that induced *S. enterica* serovar Typhimurium 4/74 phage and led to bacterial lysis (data not shown). No mitomycin C-induced bacterial lysis was detected for any of the *S. enterica* serovar Dublin isolates described in this study, and no phage DNA could be purified from mitomycin C-treated *S. enterica* serovar Dublin cultures under conditions that produced phage DNA from *S. enterica* serovar Typhimurium 4/74. Therefore, there is no evidence that SDI-1 is contained within an inducible prophage.

Distribution of SDI-1 among *S. enterica* serovars. Since SDI-1 was absent from all sequenced strains of *Salmonella*, its distribution among a larger set of strains was investigated. Genomic DNA was prepared from *S. enterica* serovar Dublin SD3246, *S. enterica* serovar Gallinarum SG9 and from 71 other *Salmonella* strains. These 71 isolates included 14 *S. enterica* serovar Dublin isolates and 30 other serovars (Materials and Methods). The DNA was used as a template in PCRs with three pairs of primers which amplify regions B, D, and F (Fig. 1 and Table 3). To confirm the presence or absence of SDI-1 in these 73 isolates, Southern blot analyses were performed. Two probes were prepared (P1 and P2) (Fig. 1) and hybridized to HindIII-digested genomic DNA. All the isolates that were positive by PCR and/or Southern blot are indicated in Table 3.

The results showed that all 15 isolates of *S. enterica* serovar Dublin have the island. Interestingly, two non-serovar Dublin isolates (*S. enterica* serovar Brandenburg and *S. enterica* serovar Duisburg) were positive in all three PCRs and on both Southern blots, suggesting they could possess the entire island. One isolate of *S. enterica* serovar Choleraesuis variant Decatur was also positive in four of the five tests. In addition, *S. enterica* serovar Heidelberg SARB24, *S. enterica* serovar Miami SARB29, and *S. enterica* serovar Muenchen SARB34 hybridized to one probe, suggesting that they might carry part of the island. Further PCRs were carried out on these six non-serovar Dublin isolates to determine the extent of the sequence similarity (Fig. 1 and Table 3). *S. enterica* serovar Duisburg was negative for PCR A only. Since one of the primers used in this PCR was outside the island, it is possible that the entire island is present but that the sequence adjacent to one end of it is different from that in *S. enterica* serovar Dublin strains. *S. enterica* serovar Brandenburg possessed much of the island, but the PCRs overlapping both ends of the island were negative, suggesting the possibility that it has inserted at a different location. The other serovars have smaller portions of the island.

Mutagenesis of sequences identified by subtractive hybridization. Transposon insertion mutants of 17 of the sequences identified in the subtraction library were generated in *S. enterica* serovar Dublin SD3246 Nal^r as described earlier (Materials and Methods) to investigate the role of these sequences in vivo. The mutants each contained unique signature tags so that they could be tracked in complex pools during infection of calves as described previously (33). The sequences mutated in this way included most of the clones not similar to mobile elements (D1 to D17) except D5 and D8. The D5 sequence was not mutated, since it is part of the same gene as D4, and the D8 sequence was deleted in the mutant with the deletion of STM3021 to STM3030 (see below). In addition, the two sequences similar to RHS elements (D18 and D19) were mutated.

Seven additional tagged mutants were prepared as follows. The sequence of clone D7 was highly homologous to the *S. enterica* serovar Typhimurium LT2 gene STM3025, and D8 was homologous to STM3022. Comparisons of LT2 with the sequenced *S. enterica* serovar Gallinarum strain 287/91 showed that *S. enterica* serovar Gallinarum lacked a region of about 11 kb, containing STM3021 to STM3030. This region includes the *stdABC* fimbrial operon. A *stdA* mutant of strain SD3246 was therefore constructed, as well as a deletion mutant lacking the whole 11 kb. Another tagged deletion mutant, lacking the SDI-1 genes showing similarity to *Enterobacter* described above (from nucleotides 6076 to 25469, encoding ORF11 to ORF32 proteins), was constructed. This mutant was designated SD3246Δ SDI-1.

The subtractive hybridization sequences similar to those encoding phage-related proteins were not all individually mutated, since many of them encoded phage structural proteins that were considered unlikely to play a direct role in virulence. Several clones contained phage regions with similarity to *S. enterica* serovar Typhimurium prophages, including ST64B and Fels-2. These phages are absent from *S. enterica* serovar Gallinarum (30, 46), but large regions of both are present in the partially sequenced *S. enterica* serovar Dublin strain. PCR analysis confirmed these regions were also present in *S. enterica* serovar Dublin SD3246 (data not shown), and as noted above, there was evidence for a Fels-2-like phage carrying *S. enterica* serovar Dublin-specific genes. Two deletion mutants

TABLE 2. Analysis of predicted ORFs on the *S. enterica* serovar Dublin SD3246 26,210-bp sequence containing SDI-1[a]

ORF	Nucleotides	G+C content (%)	Protein length (aa)	InterProScan domain(s)	Best BLASTP hit(s)	Homologue accession no.	Homologue length (aa)	% Identity (range of aa positions)
1	Start–273	44.2	>91		Bacteriophage protein STY1033 (*S. enterica* serovar Typhi CT18)	NP_455512	101	100 (11–101)
2	316–918	52.9	200		Prophage proteins (*S. enterica* serovar Typhimurium LT2 Gifsy-1 STM2620 and *S. enterica* serovar Typhi CT18 STY1034)	NP_461555, NP_455513	200	99.5 (all)
3	924–1124	52.2	66		Hypothetical protein (*S. enterica* serovar Choleraesuis SC-B67)	YP_215967	66	100 (all)
4	1127–1738	58.3	203	Phage lambda NinG	Bacteriophage protein STY1035 (*S. enterica* serovar Typhi CT18)	NP_455514	203	99.0 (all)
5	1871–2668	46.1	265	Antitermination protein	Gifsy-2 phage putative molecular chaperone STM1022 (*S. enterica* serovar Typhimurium LT2)	NP_459997	265	99.6 (all)
6	3067–3414	54.3	115	Holin	Putative bacteriophage protein STY2045 (*S. enterica* serovar Typhi CT18)	NP_456405	113	82.7 (1–110)
7	3417–4031	53.7	204	Chitinase	Lytic enzyme Sb52 (*S. enterica* serovar Typhimurium phage ST64B)	NP_700425	204	94.1 (all)
8	4028–4579	52.4	183	Signal peptide, transmembrane domain	Gifsy-1 phage gp55 precursor (*S. enterica* serovar Choleraesuis SC-B67)	YP_216199	180	94.7 (11–180)
9	4569–4982	42.0	137		None	None	NA	NA
10	5044–6018	51.9	324	Terminase small subunit	Putative phage terminase small subunit (*Klebsiella pneumoniae*)	YP_001335074	334	62.7 (1–322)
11	6008–7279	52.4	423		Putative phage terminase Ent638_1030 (*Enterobacter* sp. strain 638)	YP_001175763	402	39.9 (26–401)
12	7279–8709	51.2	476		Hypothetical protein Ent638_1031 (*Enterobacter* sp. strain 638)	YP_001175764	473	65.3 (all)
13	8681–9556	51.0	291		Hypothetical protein Ent638_1032 (*Enterobacter* sp. strain 638)	YP_001175765	274	61.3 (all)
14	9557–11131	55.7	524	NUDIX hydrolase	NUDIX hydrolase Ent638_1033 (*Enterobacter* sp. strain 638)	YP_001175766	542	54.4 (4–538)
15	11179–12024	56.1	281		Hypothetical protein Ent638_1034 (*Enterobacter* sp. strain 638)	YP_001175767	286	59.5 (9–286)
16	12042–13073	56.4	343		Hypothetical protein Ent638_1035 (*Enterobacter* sp. strain 638)	YP_001175768	343	76.9 (all)
17	13138–13623	50.6	161		Hypothetical protein Ent638_1036 (*Enterobacter* sp. strain 638)	YP_001175769	160	54 (all)
18	13636–14061	52.3	141	Signal peptide	Hypothetical protein Ent638_1037 (*Enterobacter* sp. strain 638)	YP_001175770	142	50 (all)
19	14058–14489	53.0	143		Hypothetical protein Ent638_1038 (*Enterobacter* sp. strain 638)	YP_001175771	148	74.8 (6–148)
20	14473–15411	48.9	312		Hypothetical protein Ent638_1039 (*Enterobacter* sp. strain 638)	YP_001175772	313	82.1 (all)
21	15416–16810	51.7	464		Hypothetical protein Ent638_1040 (*Enterobacter* sp. strain 638)	YP_001175773	464	77.6 (all)
22	16814–17251	52.5	145		Hypothetical protein Ent638_1041 (*Enterobacter* sp. strain 638)	YP_001175774	145	79.3 (all)

Continued on following page

TABLE 2—*Continued*

ORF	Nucleotides	G+C content (%)	Protein length (aa)	InterProScan domain(s)	Best BLASTP hit(s)	Homologue accession no.	Homologue length (aa)	% Identity (range of aa positions)
23	17251–17838	52.6	195		Hypothetical protein Ent638_1042 (*Enterobacter* sp. strain 638)	YP_001175775	192	66.8 (all)
24	17962–20016	50.9	684	Transmembrane domain	Hypothetical protein Ent638_1043 (*Enterobacter* sp. strain 638)	YP_001175776	606	68.5 (1–594)
25	20016–20513	56.4	165		Hypothetical protein Ent638_1044 (*Enterobacter* sp. strain 638)	YP_001175777	238	80.6 (1–165)
26	20729–21004	43.8	91		Hypothetical protein Ent638_1045 (*Enterobacter* sp. strain 638)	YP_001175778	91	60.6 (all)
27	21004–22056	48.1	350		Hypothetical protein Ent638_1046 (*Enterobacter* sp. strain 638)	YP_001175779	339	57.7 (1–332)
28	22053–22769	53.4	238		Hypothetical protein Ent638_1047 (*Enterobacter* sp. strain 638)	YP_001175780	238	68.1 (all)
29	22766–23098	47.1	110		Hypothetical protein Ent638_1048 (*Enterobacter* sp. strain 638)	YP_001175781	110	75.5 (all)
30	23095–24321	47.8	408		Hypothetical protein Ent638_1049 (*Enterobacter* sp. strain 638)	YP_001175782	472	52.9 (1–270), 32 (351–472)
31	24305–24931	44.5	208		Hypothetical protein Ent638_1050 (*Enterobacter* sp. strain 638)	YP_001175783	234	43.8 (1–159)
32	24928–end	49.4	>427	Phage tail collar	Hypothetical protein Ent638_1051 (*Enterobacter* sp. strain 638)	YP_001175784	232	39 (1–100)
					Gifsy-2 phage tail fiber protein STM1049 (*S. enterica* serovar Typhimurium LT2)	NP_460024	812	97 (503–670)

[a] NA, not applicable.

of the Fels-2-like phage that lacked genes STM2694 to STM2706 and STM2694 to STM2722 were generated. Bacteriophage ST64B carries the effector gene *sseK3*. An *S. enterica* serovar Dublin *sseK3* (sb26) mutant, as well as a deletion mutant lacking a large region of this phage (genes sb1 to sb25), was constructed.

Analysis of the role of *S. enterica* serovar Dublin SD3246 genes identified by subtractive hybridization in calves. A pool containing the 24 mutants described above together with 12 control strains was inoculated orally into two calves (Materials and Methods). The controls consisted of three tagged virulent *S. enterica* serovar Dublin SD3246 strains, three tagged virulent *S. enterica* serovar Gallinarum SG9 strains, three *S. enterica* serovar Dublin SD3246 type III secretion system-1 (T3SS-1) mutants, and three *S. enterica* serovar Dublin SD3246 T3SS-2 mutants. The fate of these 12 control strains had previously

TABLE 3. Distribution of SDI-1 among *Salmonella* serovars

Strain/serovar (*n*)	PCR result[a]							Southern blot analysis result[b]	
	A	B	C	D	E	F	G	P1	P2
S. enterica serovar Gallinarum SG9	ND	−	ND	−	ND	−	ND	−	−
S. enterica serovar Dublin (15)	ND	+	ND	+	ND	+	ND	+	+
S. enterica serovar Brandenburg S8	−	+	+	+	+	+	−	+	+
S. enterica serovar Duisburg S18	−	+	+	+	+	+	+	+	+
S. enterica serovar Heidelberg SARB24	−	−	−	+	+	−	−	−	+
S. enterica serovar Miami SARB29	−	−	−	+	+	−	−	−	+
S. enterica serovar Muenchen SARB34	−	−	−	+	+	−	−	−	+
S. enterica serovar Choleraesuis variant Decatur SARB70	−	−	−	+	+	+	−	+	+

[a] The PCRs amplified regions as follows (see Fig. 1): A, bases 4532 to 5804; B, 7205 to 8312; C, 9887 to 11386; D, 15483 to 16857; E, 18148 to 20265; F, 21758 to 22573; G, 24017 to 25736. ND, not determined.

[b] The probes were as follows (see Fig. 1): P1, bases 13820 to 14719; P2, bases 21758 to 22573.

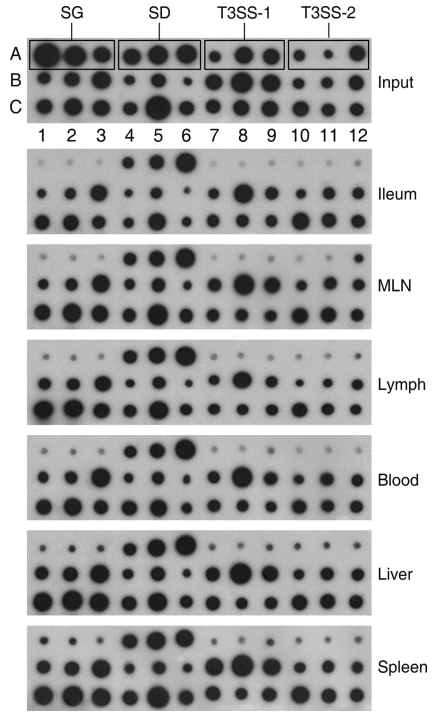

FIG. 2. Analysis of the role of *S. enterica* serovar Dublin (SD) SD3246 genes absent from *S. enterica* serovar Gallinarum (SG) SG9 in invasion of distal ileal mucosa, spread to draining MLN, lymphatic translocation, and dissemination to organs and blood. Representative blots from one calf show the prevalence of defined signature-tagged SD3246 mutants from tissues, blood, and lymph at 72 h post-oral inoculation relative to the input. Row A contains the controls as follows: wells A1 to A3, virulent tagged *S. enterica* serovar Gallinarum SG9 controls; A4 to A6, virulent tagged *S. enterica* serovar Dublin 3246 controls; A7 to A9, *S. enterica* serovar Dublin SD3246 T3SS-1 mutants; and A10 to A12, *S. enterica* serovar Dublin SD3246 T3SS-2 mutants. Rows B and C show *S. enterica* serovar Dublin SD3246 mutants prepared in this study as follows: well B1, clone D1; B2, clone D2; B3, clone D3; B4, clone D4; B5, clone D6; B6, clone D7; B7, clone D9; B8, clone D10; B9, clone D11; B10, clone D12; B11, clone D13; B12, clone D14; C1, clone D15; C2, clone D16; C3, clone D17; C4, clone D18; C5, clone D19; C6, SD3246ΔSDI-1; C7, STM3021 to STM3030 deletion mutant; C8, *stdA* deletion mutant; C9, sb1 to sb25 deletion mutant; C10, *sseK3* deletion mutant; C11, STM2694 to STM2706 deletion mutant; and C12, STM2694 to STM2722 deletion mutant.

been assessed in this model and they therefore serve as internal standards (33). Calves were anesthetized 72 h after oral inoculation, and jugular blood and efferent lymph were collected from a cannulated vessel draining the distal ileal loop as described in Materials and Methods. Biopsy specimens from distal ileal mucosa, draining MLN, liver, and spleen were collected at the end of each experiment. Duplicate dot blot hybridizations were performed with [^{32}P]dCTP-labeled tags amplified from bacteria in the input and output pools from each site. Only one of the calves had bacteremia as detected by direct plating of blood. Representative blots obtained from this calf showing the fate of mutants at each site are shown in Fig. 2. The other calf gave comparable results in all the output pools except blood, of which a representative pool could not be obtained.

The three virulent *S. enterica* serovar Dublin SD3246 controls were present in efferent lymph and all enteric and systemic tissues examined 72 h after oral inoculation. In contrast, the serovar Gallinarum SG9 controls had been cleared from all sites by this time. The T3SS-1 and T3SS-2 apparatus mutants were also recovered in smaller quantities than the input amounts at enteric sites by 3 days postinoculation and were not recovered from systemic sites or lymph. None of the other mutants appeared to be underrepresented in any of the output pools compared to the input, suggesting that their ability to invade, translocate, or persist in enteric or systemic tissues was not substantially reduced.

Functional characterization of SDI-1. To further evaluate the contribution of SDI-1 to the virulence of *S. enterica* serovar Dublin in vivo, the phenotype of SD3246ΔSDI-1 relative to the parent strain was assessed in calves in competition experiments. Six calves were orally inoculated with a mixture of equal numbers of *S. enterica* serovar Dublin SD3246 wild type (Nal^r) and SD3246ΔSDI-1 organisms. The CIs were determined at enteric and systemic sites 3 days postinfection as described in Materials and Methods (Table 4). The CIs were consistently below 1, with mean values ranging from 0.631 to 0.792 for the tissues examined. A Mann-Whitney nonparametric test indicated that the output ratios for all sites were significantly lower than the input ratio in the inocula (the *P* value was 0.0048 at all sites; Table 4). In contrast, the in vitro CI for this mutant in minimal medium was 1.12. Although the level of attenuation was modest, these results indicate that SDI-1 contributes to the pathogenicity of *S. enterica* serovar Dublin in calves.

The role of SDI-1 was next investigated using cultured-cell assays. In J774 murine macrophage-like cells, the SD3246Δ SDI-1 mutant was killed at a rate similar to the rate at which the wild type was killed (Fig. 3A). An *S. enterica* serovar Dublin Δ*phoPQ* mutant created by linear recombination was killed at a significantly higher rate than the wild type was, as expected ($P = 0.001$). The SD3246ΔSDI-1 mutant also entered INT407

TABLE 4. Competitive indices for SD3246ΔSDI-1 in calves

Site	CI for indicated calf						Mean CI (SEM)[a]
	A	B	C	D	E	F	
Ileal mucosa	0.556	0.721	0.702	0.778	0.497	0.769	0.671 (0.048)
MLN	0.663	0.822	0.904	0.794	0.785	0.786	0.792 (0.032)
Lymph	0.593	0.635	0.803	0.727	0.682	0.706	0.691 (0.030)
Liver	0.643	0.444	0.589	0.749	0.788	0.763	0.663 (0.054)
Spleen	0.434	0.581	0.724	0.725	0.603	0.718	0.631 (0.047)

[a] The Mann-Whitney test was used to determine whether the output ratio was significantly different from the input ratio at each of the five sites. A *P* value of 0.0048 was obtained at all sites.

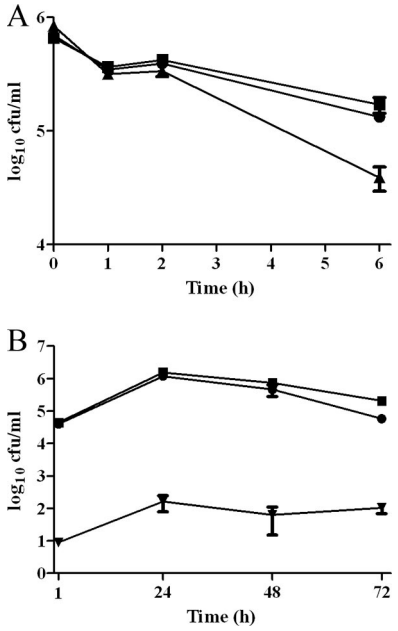

FIG. 3. Interaction of *S. enterica* serovar Dublin SD3246 wild-type and mutant strains with cultured cells. (A) Survival in J774 cells. The symbols for strains are as follows: ●, wild-type Nal^r strain; ■, SD3246ΔSDI-1; and ▲, *phoPQ* mutant. (B) Invasion, intracellular growth, and survival in INT407 cells. The symbols for strains are as follows: ●, wild-type Nal^r strain; ■, SD3246ΔSDI-1; and ▼, *sipD* mutant. Data points represent the means ± standard errors of three or four independent experiments, with triplicate wells.

intestinal epithelial cells in numbers similar to those of the parent strain (1-h time point; Fig. 3B) and intracellular replication was comparable at 24 h. As expected, an *S. enterica* serovar Dublin SPI-1 (*sipD*) mutant was substantially impaired in its ability to invade INT407 cells. By 3 days postinfection, SD3246ΔSDI-1 was recovered in numbers that were significantly higher than those of the wild type ($P = 0.014$). Cytotoxicity induced by these two strains was not significantly different as determined by a lactate dehydrogenase release assay (data not shown). Taken together with the CI during growth in minimal media, these data indicate that the SDI-1 mutation does not exert a fitness cost per se.

To investigate whether SDI-1 is a host-specific virulence factor, we also performed competition experiments with inbred mice. A dose of ca. 2×10^6 CFU comprising equal amounts of SD3246ΔSDI-1 and the parent strain was given to 16 female C57BL/6 mice by oral gavage. Animals were humanely killed upon presentation of symptoms of salmonellosis, and homogenates of spleen and liver were plated for the determination of CIs. Bacteria were recovered from both sites in all mice (at least 7×10^5 CFU). Four mice that presented disease at 3 to 4 days postinoculation had mean CIs of 1.01 ± 0.07 and 1.17 ± 0.12 in the spleen and liver, respectively. The CIs in the remaining mice that presented disease at later time points (up to 6 days postinoculation) were more variable, possibly owing to a bottleneck in the establishment of persistent infection in mice of this type with SD3246 at this dose. Nevertheless, for the majority of mice, the CI was >1 (9 of 12 spleen samples and 8 of 12 liver samples), with 6 of the 12 mice yielding only the mutant strain at post mortem examination. Taken together, these data indicate that SDI-1 is not required for virulence in mice and imply that it may play a host-specific role in *S. enterica* serovar Dublin pathogenesis in cattle.

DISCUSSION

The bacterial and host factors that determine why some *S. enterica* serovars translocate to distal sites while others are restricted to the gastrointestinal tract are ill defined. We previously showed that the ability of *S. enterica* serovar Dublin to persist in bovine ileal mucosa and translocate via efferent lymphatics compared with other serovars in cattle correlated with systemic virulence (28, 33). In contrast, the systemic virulence of host-restricted serovars did not correlate with intramacrophage survival (44) or with invasion or damage of the ileal mucosa (9, 27, 28, 42) but did correlate with reduced net replication in the intestinal wall and with reduced inflammation in the ileum (27). It has been shown that *S. enterica* serovar Typhi reduces Toll-like receptor-dependent interleukin-8 expression and subsequent inflammation in the intestinal mucosa by a process requiring the Vi capsular antigen (34). These findings suggest that the greater induction of proinflammatory responses by rapidly proliferating ubiquitous serovars might result in them being confined to the intestines, whereas host-restricted and host-specific serovars may have developed mechanisms to evade or suppress activation of host innate immunity at mucosal surfaces and thus disseminate to distal sites.

The screening of mutant banks of the ubiquitous serovar *S. enterica* serovar Typhimurium has shown that different genes are utilized to colonize different animal hosts (8, 25, 40). The repertoire and sequence of such factors have the potential to influence host and tissue tropism. For example, it has been established that different serovars express different sets of fimbrial operons (reviewed in reference 13) and that adaptation to the avian host is often associated with the loss of type 1 fimbriae and motility (10, 23). It has also been suggested that factors involved in host restriction may have a metabolic basis. For example, *S. enterica* serovar Dublin is a nicotinic acid auxotroph, and it is interesting that cattle can synthesize nicotinic acid and do not require niacin in their diet (16).

In this study, we have identified genes present in *S. enterica* serovar Dublin SD3246 but not *S. enterica* serovar Gallinarum SG9 to dissect the genetic basis of the differential virulence of these two strains in cattle. It is difficult to estimate the percentage of *S. enterica* serovar Dublin-specific sequences that we have identified here, due to the absence of a complete *S. enterica* serovar Dublin genome sequence. Our library identified three fragments totaling approximately 2 kb on the 21-kb SDI-1. If this is representative, it suggests coverage of just under 10%. We identified a total of 41 *S. enterica* serovar Dublin SD3246 DNA sequences that were absent from *S. enterica* serovar Gallinarum SG9. As expected, many of these corresponded to mobile elements, particularly prophage genes. Another group was present in a range of serovars, including ubiquitous serovars, and some of the differences had previously been reported (30). For example, *S. enterica* serovar Dublin SD3246 fragments D1 and D2 (Table 1) corresponded to the *Salmonella* microarray region B16, SD3246 fragment D13 was

on microarray region A02, and fragment D6 was on microarray region B08 (30). These regions had been shown, by use of the microarray, to be present in *S. enterica* serovar Dublin but absent from some *S. enterica* serovar Gallinarum isolates (30), which is consistent with our findings.

Of particular interest were the sequences that were unique to *S. enterica* serovar Dublin. Further analysis of these showed that two of the unique sequences, D14 and D15, were on a Fels-1-like prophage, and two others, D16 and D18, were associated with RHS genetic elements. The roles of these four regions are unknown, but these fragments all had very low G+C contents, indicating that they may have been acquired relatively recently by lateral gene transfer. Interestingly, a number of *Salmonella* virulence factors located on prophages have previously been described; for example, *S. enterica* serovar Typhimurium LT2 has four prophages that all carry one or more genes involved in virulence, such as *nanH* and *sodCIII* on the Fels-1 prophage (14). Also, the horizontally acquired SPI-6, which potentially carries a T6SS and the *saf* fimbrial operon, contains an RHS element (17, 31).

Comparisons with the databases suggested that D11 and D12 might also be specific to *S. enterica* serovar Dublin. Analysis of the flanking regions showed that these were carried on a 21-kb genomic island designated SDI-1. This island was present in all *S. enterica* serovar Dublin isolates studied, and its sequence was very highly conserved between isolates SD3246 and CT02021853. Such high sequence conservation between different isolates of host-restricted serovars, such as *S. enterica* serovar Dublin, has been noted previously (36). SDI-1 was absent from most other serovars. Exceptions included single isolates of *S. enterica* serovar Duisburg and *S. enterica* serovar Brandenburg. Analysis of the SDI-1 sequence gave few clues about the potential function of genes on the island. Although SDI-1 was flanked by phage sequences, *S. enterica* serovar Dublin isolates did not contain inducible prophages, suggesting that insertion of the island into a prophage may have disrupted the phage. SDI-1 ORF11 to ORF32 proteins were similar at the amino acid level to predicted proteins of *Enterobacter* sp. strain 638, an endophytic strain which was isolated from a plant. However, the level of nucleotide similarity was very low, so the island is unlikely to have recently originated from *Enterobacter*. The evolutionary origin of SDI-1 remains unknown.

Other regions which might be involved in the virulence or host restriction of *S. enterica* serovar Dublin were those that had very limited distributions among serovars, such as D4 and D5, which were found only in *S. enterica* serovar Dublin and *S. enterica* serovar Paratyphi A. The translated D4 and D5 sequences were highly similar to the N terminus of the *S. enterica* serovar Paratyphi A gene product, SPA2350. Although the N-terminal region of this protein is unusual, the C terminus is highly conserved in a number of *S. enterica* and *E. coli* proteins that are predicted to be autotransporters and/or virulence factors.

Screening of a pool of defined signature-tagged mutants with insertions in the subtractive hybridization library sequences in a calf model did not identify any attenuated mutants. The method confirmed attenuation of SG9 and SD3246 tagged SPI-1 and SPI-2 mutant strains detected previously (33). However, subtle attenuating effects could not be ruled out. Indeed, a competition experiment comparing the SDI-1 deletion mutant with the parent wild-type strain showed that this mutant was outcompeted by the wild type at all sites tested. This suggests the mutant colonized or persisted less well than the wild type in vivo. The CIs obtained were between 0.434 and 0.904, showing that the attenuation was less than that previously observed for T3SS-1 mutants in calves (which had CIs below 0.1 in efferent lymph and MLN 12 h after instillation into ligated ileal loops) (33). However, the CIs were consistent and the attenuation was statistically significant. Since it is likely that a number of genes are required for host adaptation (40), it is perhaps not surprising that the inactivation of one region caused such modest attenuation. No defects in invasion of cultured epithelial cells or intramacrophage survival could be detected for the SDI-1 mutant relative to the parent in assays that confirmed the known attenuating effect of SPI-1 or PhoPQ mutation. Taken together with the CI during growth in minimal medium, these data imply that SDI-1 mutation does not compromise fitness. In a murine model, when signs of systemic salmonellosis first appeared, mean CIs in the spleen and liver exceeded 1. While at later time points CIs were more variable, the mutant strain predominantly outcompeted the wild type, indicating that SDI-1 is not required for virulence in mice and may play a host-specific role in cattle.

This is the first report of an *S. enterica* serovar Dublin-specific locus that contributes to virulence in the bovine host. Further studies will be required to determine whether any of the other *S. enterica* serovar Dublin-specific regions identified here play subtle roles in host adaptation and virulence. It is also likely that other genetic mechanisms not examined here, such as gene deletions, differential expression of orthologous genes, or allelic differences in orthologous sequences, contribute to the systemic virulence of *S. enterica* serovar Dublin. However, the finding that SDI-1 plays a role in *S. enterica* serovar Dublin virulence implies that host restriction (and more severe disease outcomes) may not be solely due to gene decay but may require the acquisition of specific factors.

ACKNOWLEDGMENTS

This work was supported by the Biotechnology and Biological Sciences Research Council and the Department for the Environment, Food and Rural Affairs (grant C50964X).

We thank D. Prickett and M. Watson for bioinformatics support and D. Hudson and G. Prescott for technical assistance.

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Editor: A. J. Bäumler